United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Comparative toxicity of microcystin congeners in mice and age-related factors as toxicity determinants
LAPR Number:	18-09-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 RTP/USEPA/US
Date Originated:	08/13/2015
LAPR Expiration Date:	09/30/2018
Agenda Date:	09/23/2015
Date Approved:	10/02/2015
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 /USEPA/US by Exemption 6 /RTP/USEPA/US	10/02/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	10/01/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Comparative toxicity of microcystin congeners in mice and age-related factors as toxicity determinants

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
SSWR 6.02D Development of approaches to evaluate human health responses to water-borne contaminant associated with drinking water quality

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/TAD/DTB/[redacted]-2012-01-r02

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** [redacted]/RTP/USEPA/US	TAD ***Branch*** DTB	MD B105

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** [redacted]TP/USEPA/US	TAD ***Branch*** DTB	MD B105

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the

continuation. Please spell out all acronyms and abbreviations with their initial use.

The primary objective of the studies proposed here is the characterization of the comparative toxicity of a series of microcystins given either by oral or intraperitoneal (i.p.) route to CD-1 mice. A secondary objective is to investigate body composition as a possible factor in the apparent increased susceptibility of aged animals to the toxic effects of microcystins.

Microcystins (MCYSTs) constitute a family of related heptapeptides - 7 different amino acids arranged, in part, in a ring structure. These toxins are produced by cyanobacteria ("blue-green algae") and are found globally. They are the most common toxins found in freshwaters in the United States. There are over 60 different structural varieties (congeners) that often fundamentally differ in the identity of one or two amino acids in the ring. The most common type of MCYST is microcystin-LR (MCLR), a form with the two variable amino acids being leucine (L) and arginine (R). Other congeners of microcystin differ in the identity of these two variable amino acids - for example, MCRR has two arginines and MCLF has a leucine and a phenylalanine (F). In 2014 MCLR caused Toledo, OH, drinking water to be declared not safe for consumption because levels exceeded the WHO Safety Guidelines for the toxin. There are other toxins found in U.S. freshwaters and these can be the dominant forms in some algal blooms. A small number of in vitro and intraperitoneal (i.p.) studies have been used to investigate the acute toxicity of a small number of congeners that have been identified in U.S. lakes and the data indicate considerable differences in inter-congener toxicity. Common analytical techniques measure total MCYSTs without distinguishing between them. Regulatory decisions for safety in both recreational and drinking waters are made with the total MCYST value assumed to be similar in toxicity as MCLR. This does not consider the possibility of other MCYST congeners with differing toxicity being present and altering the actual MCYST toxicity as compared to similar levels of MCLR. The possibility of either underestimating or overestimating the actual toxicity of waters requiring a regulatory decision, is a potential problem that the Office of Water considers to be important. The absence of MCYST toxicity data using the oral route of exposure is especially problematic since this is the most relevant route of exposure. The studies in this LAPR will address the issue of comparative MCYST congener toxicities done with similar protocols in the same laboratory, and more importantly, will use the oral route of administration in addition to the much more common intraperitoneal route – the paucity of oral exposure data is because of its much lower toxicity as compared to the i.p. route (≈100-fold less toxic) coupled with the difficulty in obtaining the required amounts of any congeners with the exception of MCLR and MCRR.

This proposal is for the evaluation of six MCYST congeners, all of which have been found in U.S. recreational waters: MCLR, MCRR, MCLA, MCYR, MCLY, and MCLF. Most of the existing information is derived from a single or a very small number studies. There is also a general lack of information on the half-life of these toxins in mammals. It is therefore necessary to use the in-vivo model as a basis for the formulation of regulatory decisions that may be based, in part, on potential health concerns. The comparative toxicity data in this proposal should allow the E.P.A. to make decisions on simultaneous exposures to multiple congeners using a scientific rationale based on data from animals exposed via the appropriate oral route. It may also enable a better general comparison of the oral and i.p. routes of administration which may be extremely useful given that the i.p. route will continue to be used because of the reasons mentioned above.

The secondary objective concerns replicated data that appear to indicate MCLR toxicity is greater in aged as compared to younger animals. These findings are most often based on overt toxicity from acute exposures and contain few, if any, clinical chemistry, tissue level or histological endpoints. The reasons for this age-related susceptibility are generally considered to be related to differences in metabolism, distribution and/or excretion rates, but there is little evidence supporting any of these hypotheses. We will investigate a possible factor that has not, as yet, been considered – the possibility that the reported increased susceptibility is not due to age-related changes in pharmacokinetics, but is due to an increased body fat load in aged animals. This hypothesis may be supported by the fact that older mice, especially males, gain a great deal of weight – going from 25-30g. at puberty to 50g. at 6-8 months. Much of this increased weight is due to changes in body composition as simple observations at necropsy and our body composition data indicate. MCYST congeners are extremely hydrophilic ("water loving"), as are most algal toxins and they will not be closely associated with body fat. Most dosing of animals is done on a body weight basis with no allowance for body composition changes. Dosing an animal with high fat content with a highly hydrophilic compound like any MCYST congener may significantly increase the dose to the "lean" part of the animal thus resulting in a much higher effective dose as compared to an animal that weighs much less because of significantly less body fat content. Aged mice, especially males, tend to get obese and may therefore be exposed to higher effective doses, and show a greater

toxic response – not due to metabolic alterations, but due to their obesity. We therefore propose to test the toxicity of MCLR in animals of the same age with different body compositions and of different ages with similar body composition (see attached flow chart). Data analyses should enable us to assign a relative contribution of age and body fat content to the observed toxicity. If the results of these studies indicate that body composition influences individual toxic responses to MCLR, we will repeat the studies using a hydrophilic algal toxin, cylindrospermopsin (CYN), that should yield similar types of results and a lipophilic toxin, okadaic acid ("diarrhetic shellfish poison") where increased fat content should have the opposite effect on toxicity.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

There is no computer model or validated in vitro bioassays system that can accurately predict toxicity from exposures
via the oral route. Extremely little is known about four of the six congeners that will be studied and it is not possible to make accurate predictions of the i.p. or in vitro toxicity of these four solely using data from MCLR in vivo studies. Animals are therefore necessary to allow for the generation of a data base such as the one proposed here.

b. Justify the species requested:

Studies to evaluate the toxicity of algal toxins have utilized the mouse in the vast majority of instances. The reasons for the use of the mouse rather than the rat are based on the relative size of these two species. The difficulty in obtaining sufficient toxin to study in the rat has limited its use to a very small number of studies that have not used the oral route. In addition, the physiology of the mouse is well characterized, including a considerable knowledge of the structure and function of the liver that is the target organ. Finally, our laboratory has regularly utilized the mouse model in studies involving the types of dosing regimens and experiments covered by this LAPR.

3. How was it determined that this study is not unnecessary duplication?

We have searched the literature listed in PubMed from 1980 to the present using keywords “microcystin-LR”, microcystin-RR, “microcystin-LA”, “microcystin-YR”, “microcystin-LY”, "microcystin-LF”, and “toxicity”, as well as "aged animals", "microcystin-LR", "cylindrospermopsin", "okadaic acid", and "domoic acid". The literature searches indicate that there are no in vivo studies for any congeners except MCLR and MCRR and the experimental endpoints in those are more limited in scope than the ones we will use; and that the effects of aging on the toxicity of both CYN and domoic acid have not been studied.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

The animals used will be equal numbers of male and female CD-1 mice, 5-7 weeks of age, in both studies. Mice will be acclimated for a week before exposures.
All dosing or diet groups will have equal numbers of male and female animals.

The endpoints for all of the studies will consist of:
Observational (activity, responsiveness, appearance, weight change, and organ weights and appearance at the time of necropsy).
Clinical chemistries using serum collected at necropsy (enzyme indicators of hepatic toxicity, protein, glucose, indicators of bile function, creatinine, BUN).
Real-Time PCR to assess known gene expression indicators of hepatic toxicity (e.g. Bax, p53, RPL12a).
Additional endpoints that we will collect tissues for include:
Histopathology on liver tissue.
Levels of MCYST congeners in liver determined by LC/MS analyses.

Comparative toxicity studies:
Animals - Male and female CD-1 mice will be used.
Dosing period - Three consecutive days. The rationale for this exposure period is based on 2 factors:
1. Most MCYST exposures to humans in the U.S. would involve a pulse exposure either through recreation or

food.
2. The toxic effects of single acute and repeat dose exposures are not the same – acute doses of MCLR involve rapid onset of internal bleeding, resultant congestion of blood in the liver and significant adverse hemodynamic effects, while multiple doses work more slowly and endpoints center on hepatotoxicity.
Route of exposure - The routes of exposure will be oral and i.p. Oral dosing is the central endpoint, but i.p. dosing is necessary for a better understanding of the strengths and limitations of the i.p. route in the regulatory environment. This is necessary since most studies will continue to be i.p. due to difficulty obtaining sufficient amounts of many congeners for use in oral route studies. These data may be relevant to the important question of whether the ratio of i.p. and oral toxicity remains constant for different congeners.

Doses and numbers of animals – Preliminary Studies:
Since we can't assume that the relationship of MCLR acute oral and i.p. dose levels are applicable to all of the congeners on study we will do a pilot/preliminary study. It will use 2 treated animals + 2 controls for each congener at doses of 40ug/kg, i.p. and 4mg/kg oral that our data and published studies indicate should be at, or slightly below, an exposure inducing some degree of overt toxicity. This will require 4 mice per dose route, i.p. or oral x 6 congeners: 4 mice x 2 routes x 6 congeners = 48 mice.

– Definitive Studies:
The definitive studies will use a total of 10 animals for each dose group and controls (see B.2. for justification). Both i.p. and oral route studies will be used. There will be at least 2 dose levels: a higher dose level (approx. 40ug/kg, i.p. and 4mg/kg oral) that our data and published studies indicate should be at, or slightly below, an exposure inducing some degree of overt toxicity, and a lower dose level (approx 30ug/kg, i.p. and 3mg/kg oral) that should only induce minor effects.

Data collection – Animals will be observed during the dosing period (see Section B.6.e for details). We will euthanize all animals 24hrs after the last dose.
Animal numbers – 10 animals will be used for each dose and control group.

Age-Diet study:
Animals – male and female CD-1 mice.

Preliminary study:
We will use 120 animals (60 males and 60 females) to obtain weight, body composition measurements, and serum chemistries. The endpoint in this effort is to identify time points where body composition measures differ significantly between the Harlan control diet and Harlan High Fat (HF) diet (TD06414) populations. Body composition and weight measurement will be obtained at bi-weekly intervals. After an acclimation period of 1 week animals will be placed on either control or HF diets. Three time points for necropsies will take place:
1st. Immediately prior to being placed in either control or HF diets.
2nd. At the time point where the body composition becomes significantly different in the control and HF diet animals. This should occur at 3-4 months.
3rd. At the time point where animals on the control diets reach the same body composition as the HF animals did at the time of the second euthanization. This should occur at 6-8 months. It should be noted that the 2nd and 3rd time estimates are based on findings in other laboratories and our time points may differ.
These time points will be used for the definitive study since they will allow us to compare MCLR toxicity in populations of animals with similar age and different body compositions (2nd euthanization) and similar body compositions and different ages (2nd euthanization HF animals and 3rd euthanization controls) (see attached flow chart). We will also have data from beginning of the study (1st euthanization) that will give us baseline values, and HF animal data from the 3rd euthanization that may give additional indications of change due to fat deposition over time. Each necropsy point will involve 20 animals for each diet type (control and HF). Serum will be obtained to obtain baseline data useful for further assessments of changes in liver enzymes indicators of hepatic dysfunction, protein, glucose, indicators of bile function, creatinine, and blood urea nitrogen (BUN).

Definitive study:
Design – Animals will be placed on either Harlan lab chow (control diet) or Harlan High Fat diets. At the three

time points obtained in the preliminary study, animals will begin to be dosed with MCLR for three consecutive days - 1 week after acclimation and the two additional time points obtained in the preliminary study. All animals will be euthanized 24hrs after the third and final dose and the endpoints listed above will be evaluated.
Dosing period – Three consecutive days.
Route of exposure - The i.p. route will be used.
Doses and numbers of animals – At least two dose levels will be used, a high level of MCLR (40ug/kg) expected to cause signs of toxicity and a low dose (30ug/kg) that should only cause minor effects. A minimum of 20 animals receiving either control of HF diet will be dosed with MCLR or sterile water (control group) and necropsied at each time point.
Data Collection - Animals will be monitored at weekly intervals and growth measured. After euthanization, we will collect tissues and blood for the assays listed above.
Animal numbers - At all three time points we will euthanize 20 animals for each dose level and controls. These animals will have blood collected for clinical analyses and tissues of interest for MCLR levels and possible histopathology.

	Comparative-Prelim	Comparative-Defin	Age-Prelim	Age-Defin
Animals/group	2	10	20	20
MCYST congeners - toxins	6	6	-	1
Routes	2	2	-	1
Dose groups (incl. controls)	2	3	1	9
Sacs	1	1	3	3
Diets	1	1	2	2
Total	48	360	120	1080
Control - Treated	24 - 24	120 - 240	120 - 0	360 - 720

A future Amendment will be submitted to enlarge the scope of the work wil be submitted if the results of these studies indicate that body composition plays a major role in the response to MCLR, we propose to use an additional hydrophilic algal toxin, CYN, to test the generality of the role that levels of body fat have on the response of mammals to hydrophilic algal toxins. The definitive study will be repeated with CYN and doses of 40ug/kg and 30ug/kg I.p. will be administered for three consecutive doses as in the MCLR study. The higher dose should produce toxicity while the lower dose should induce only minor effects. If the data for these two algal toxins are similar in relation to body composition, a third algal toxin, okadaic acid that is lipophilic, will be used in this experimental design. Dose levels for okadaic acid will be 150ug/kg and 100ug/kg, the higher dose should produce both hepatic injury that will be evaluated by serum indicators of hepatic injury, and gastrointestinal tract injury that will be evauated using histopathologic analysis. The lower dose level should induce only minor effects.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.
A minimum of 10 animals per group will be used throughout the comparative toxicity studies. The rationale for this minimum group size is based on a power calculation done on data involving MCLR clinical chemistry responses. The statistician found that a minimum of 10 animals will be necessary to be able to detect a $p<0.05$ in 80% of the data sets.
We will use 20 animals/sex/dose group for the age-diet studies. Larger numbers will increase our ability to find significant changes in populations of treated animals that have a high degree of inter-animal variability.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	624	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	984	

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
I.p. injections of sterile water; 0.1ml total volume; 26 guage needle; three consecutive days.
Gavage of sterile saline; 0.2ml total volume; standard mouse feeding needle; three consecutive days.

b. Survival Blood Collections (method, volume, frequency):
na

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Animals will be placed on Harlan control diet except for the age-diet study that will, in addition, also use Harlan High Fat Diet (#TD06414).

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
na

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
na

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:
Animals will be housed 3 or 2/cage - all cages will be numbered and all animals will receive ear punches. The combination of cage number and ear punches will allow identification of individual mice for the duration of the study. Animals will be examined for 1hr post initial dosing and thereafter at hourly intervals during for the rest of the work day. For the 2nd and 3rd doses the animals will be observed for the 1st hour post dosing and thereafter at 2hour intervals during the work day. If any toxicity is observed after dosing, the times between observations will be shortened. Animals will be weighed daily. All observations will be done by study personnel; **Exemption 6**
Exemption 6Exemption 6

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):
I.p. injections of 40ug/kg or 20ug/kg MCYSTs in sterile water; 0.1ml total volume; 26 guage needle; three consecutive days.
Gavage of 4mg or 3mg/kg MCYSTs in sterile saline; 0.2ml total volume; standard mouse feeding needle; three consecutive days.

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):
terminal blood will be collected at euthanization times. The technique will involve cardiac puncture after CO2 anesthesia and wil be immediately followed by exanguination.

c. Testing methods:
Animals will be placed on Harlan control diet except for the age-diet study that will, in addition, also use Harlan High Fat Diet (#TD06414).

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):
na

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):
Animals will be housed 3 or 2/cage - all cages will be numbered and all animals will receive ear punches. The combination of cage number and ear punches will allow identification of individual mice for the duration of the study. Animals will be monitored for the 1/2hr post initial dosing and thereafter at hourly intervals

during after the first day until 6pm. For the 2nd and 3rd doses the animals will be observed for the 1st 1/2hr and at 1hr post dosing and thereafter at 2hour intervals until 6pm. If any toxicity is observed after dosing, the times between observations will be shortened. Animals will be weighed daily. All observations will be done by study personnel Exemption 6Exemption 6Exemption 6

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

Analgesics will not be used because the interaction of analgesics with MCYSTs is unknown. The necessary study comparing treated animals with and without analgesics would actually lead to the use and eventual euthanasia of another set of animals. Also, the response to toxicity involves initiation of the stress cascade that, in itself, alters numerous normal responses to xenobiotics - the absence of the stress response would therefore create a situation that would make extrapolation to human or other animal populations more difficult.

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

Treatment-related deaths are not expected.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

na

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

na

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

na

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

na

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

na

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Animals will be monitored daily as detailed above in Sections 5f and 6e. The PI, Exemption 6 and the study veterinarian, Exemption 6 will be responsible for monitoring. We do not expect to see rapid substantial overt toxicity including lethargy, hunching, difficulty breathing or other signs of morbidity, but given that most of the MCYST congeners have not been studied in any detail and the oral route has only been used for 2/6 congeners, the possibility of unexpected toxicity does exist. Similarly, we do not expect to see significant overt toxicity including the above observations as well as diarrhea, or weight loss >10% of body weight, in animals after the first day. In the event of any the above listed toxin-induced effects, the Attending Veterinarian will be notified immediately.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Any animals displaying non-responsiveness to interaction, inappetence, lethargy, hypothermia, diarrhea, and/or weight loss greater than 10% will be removed from the study, euthanized, necropsies performed and blood and tissues collected for analysis as needed. Animals exhibiting signs of morbidity will be immediately euthanized and necropsied.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

We have searched the literature listed in PubMed from 1980 to the present using keywords “microcystin-LR”, microcystin-RR, “microcystin-LA”, “microcystin-YR”, “microcystin-LY”, "microcystin-LF”, and "analgesics" did not identify any studies in which analgesics had been used in conjunction with these toxins. Analgesics will not be used, therefore, because the interaction of analgesics with these toxins is unknown.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:	1608
b. Animals to be transferred from another LAPR:	0
LAPR Number that is the source of this transfer:	
c. Animals to be transferred from another source:	0
d. Offspring produced onsite (used for data collection and/or weaned):	0
e. TOTAL NUMBER of animals for duration of the LAPR	1608

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: CD-1 mouse/mice

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

na

4. Sources of animals:

Charles River Laboratories

5. Provide room numbers where various procedures will be performed on animals:

Animal holding rooms for dosing and surgical suites for necropsies and tissue collections.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

Exem Exem Exem *Room Numbers* Except Except Except

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

na

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

na

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

na

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

Animals will be group housed (two or three per cage) on heat-treated pine shavings in solid-bottom polycarbonate cages. An igloo or tube and Enviro-Dri may be added for enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.

Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

Microcystin congeners: MCLR, MCRR, MCLA, MCYR, MCLY, and MCLF; cylindrospermopsin and okadaic acid are all covered under HSRP164. The LD50s for the congeners are as follows (i.p. data in ug/kg); MCLR 50; MCRR 500-800; MCLA 50; MCYR 150-200; MCLY 90; MCLF unknown. The i.p. LD50's for CYN and okadaic acid are 50ug/kg and 200ug/kg respectively. The maximum doses administered will be 40ug/kg for CYN, 150ug/kg for okadaic acid, and 40ug/kg for the MCYST congeners. It should be noted that the toxicity values for the MCYST congeners are based on a small number of studies and in those cases where there is more than one paper listing such data, there is wide variability. For example, MCRR is listed as 10-15-fold less toxic in one paper and 3-fold less toxic in another. Some of these differences may be due to different mouse strains having been used in these studies, but older papers may not have had proper analysis of the toxins used and the doses delivered may not be accurate. The maximum doses of the congeners may have to be adjusted up or down based on our preliminary studies. Also, we are confident that the CYN doses we propose to use will induce the toxicity we expect. This toxicity will include alterations in serum enzymes indicative of hepatic toxicity (e.g. SDH, LDH, ALT), gene expression changes (Bax, Trp53), mild necrosis in the centrilobular region of the liver), but we have not used okadaic acid and although the literature is consistent concerning LD50 data, there is relatively little lower dose data and, again, we may have to adjust our doses based on the results we obtain.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

None of the test compounds are available as "pharmaceutical grade". We will use the purest materials that we are able to obtain.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

none

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

None of these toxins are volatile and normal handling procedures - use of approved laboratory gloves, lab coats and safety glasses - will suffice.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:
na

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Study planning, animal dosing, assistance with tissue collection.	35+ years as a research toxicologist. All NHEERL required training is complete.
Exemption 6	Associate Principal Investigator	Study planning, animal dosing, tissue collection, animal evaluations, data management.	20+ years as a practicing small animal veterinarian. Five years experience with toxiccology studies involving rodents. All NHEERL required training is complete.
Exemption 6	Post-Doc	Animal dosing, assistance with tissue and blood collection, data management and analysis.	Eight years of experience using animals in research. All NHEERL required training is complete.
Exemption 6	Student	assistance at necropsies, body comp instrument, and data analysis	All relevant animal use NHEERL training courses completed.
Exemption 6	Student	Assistance with dosing, assistance at necropsies, and data analysis	All relevant animal use NHEERL training courses completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year na

2. Breeding protocols and recordkeeping na

3. Methods for monitoring genetic stability na

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR na

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?
Animals will be euthanized 24 hours after the final dose.

2. Describe the euthanasia techniques:
Method(s): Euthanasia plus exsanguination
Agent(s): CO2
Dose (mg/kg): To effect
Volume:
Route: Inhalation

Source(s) of information used to select the above agents/methods:
2013 AVMA Guidelines on Euthanasia.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).
na

4. Describe how death is to be confirmed.
Vital organ section, Prolonged absence of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Euthanized as above
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.

6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	09/02/2015

Submitted: 09/02/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	09/02/2015 by Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 /RTP/USEPA/US	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 /RTP/USEPA/US	TAD ***Branch*** RTB	MD ***Submitted to Branch Chief for Approval*** 09/02/2015 01:17 PM

ATTACHMENTS

Flow chart for MCYST LAPR.pptx 18-09-002 LAPR PI Resp.pdf

Actions

First Update notification sent: 07/27/2016
Second Update notification sent:
First 2nd Annual notification sent:
08/07/2017
Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: